THE EFFECTS OF THYROID HORMONE LEVEL AND ACTION IN DEVELOPING BRAIN: ARE THESE TARGETS FOR THE ACTIONS OF POLYCHLORINATED BIPHENYLS AND DIOXINS?

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Alterations in thyroid hormone level or responsivity to thyroid hormone have significant neurologic sequelae throughout the life cycle. During fetal and early neonatal periods, disorders of thyroid hormone may lead to the development of motor and cognitive disorders. During childhood and adult life, thyroid hormone is required for neuronal maintenance as well as normal metabolic function. Those with an underlying disorder of thyroid hormone homeostasis or mitochondrial function may be at greater risk for

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2. Abbreviations: ATP, adenosine triphosphate; cAMP, cyclic adenosine monophosphate; cDNA, complementary deoxyribonucleic acid; FB, fast blue; MAPs, microtubule associated proteins; mRNA, messenger ribonucleic acid; PCBs, polychlorinated biphenyls; PND, postnatal day; RNA, ribonucleic acid; RTH, resistance to thyroid hormone; SCH, sporadic congenital hypothyroidism; SNE, subacute necrotizing encephalomyopathy; T_3, triiodothyronine; T_4, thyroxine; TRH, thyrotropin releasing hormone; TSH, thyroid stimulating hormone; TSHr, thyroid stimulating hormone receptor.

3. Key words: ATPase 6, dioxins, hypothyroidism, hyt/hyt mouse, mitochondria, mutation, neurodevelopment, polychlorinated biphenyls, thyroid hormone, TSH receptor.

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developing cognitive, motor, or metabolic dysfunction upon exposure to substances which alter thyroid hormone economy. Polychlorinated biphenyls (PCBs) and dioxins have been argued to interfere with thyroid hormone action and thus may affect the developing and mature brain.

Animal models provide useful tools for studying the effects of thyroid hormone disorders and the effects of environmental endocrine disruptors. The congenitally hypothyroid, hyt/hyt, mouse exhibits abnormalities in both the cognitive and motor systems. In this mouse and other animal models of thyroid hormone disorders, delayed somatic and reflexive development are noted, as are permanent deficits in hearing and locomotor and adaptive motor behavior. This animal's behavioral abnormalities are predicated on anatomic abnormalities in the nervous system. In turn, these abnormalities are correlated with differences in neuronal structural proteins. In normal mice, the expression of mRNAs coding for these proteins occurs temporally with the onset of autonomous thyroid hormone production. The hyt/hyt mouse has a mutation in the thyroid stimulating hormone receptor (TSHr) gene which renders it incapable of transducing the TSH signal in the thyrocyte to produce thyroid hormone. Some behavioral and possibly some biochemical abnormalities in mice exposed to PCBs are similar to those seen in the hyt/hyt mouse.

In addition to direct effects on brain development and neuronal maintenance, thyroid hormone is necessary for maintaining metabolic functioning through its influence on mitochondria. Because the brain is particularly sensitive to inadequate energy generation, disorders of thyroid hormone economy also indirectly impair brain functioning. Alterations in thyroid hormone level result in differing expression of mitochondrial genes. Mutations in these mitochondrial genes lead to well-recognized syndromes of encephalomyopathy, myopathy, and multisystem disorder. Hence, PCBs and dioxins, by possibly altering the thyroid hormone milieu, may alter the functioning of mitochondria in the generation of adenosine triphosphate (ATP).

The use of animal models of thyroid hormone deficiency for behavioral, anatomic, histologic, and molecular comparison will help elucidate the mechanisms of action of these putative endocrine-disrupting compounds. The study of thyroid hormone disorders provides a template for relating thyroid hormone mediated effects on the brain to these compounds.

INTRODUCTION

Alterations in thyroid hormone level and/or responsivity to thyroid hormone have significant behavioral, neurologic, and neuropsychologic sequelae throughout the life cycle. The broad spectrum of clinical abnormalities observed in thyroid hormone disorders is related to the type of disorder, the age of onset, the duration and severity of the altered thyroid hormone level, the rapidity and efficacy of correction of the abnormality, and maintenance of adequate therapy. PCBs and chlorinated dibenzo-*p*-dioxins (dioxins) are a group of halogenated compounds that may act as neurotoxicants, fetal teratogens, or thyroid hormone antagonists. These compounds

are structurally similar to thyroid hormone(s) and may mimic or block their action. As such, these agents may contribute to the broad spectrum of clinical, neurologic, and behavioral abnormalities observed in disorders of thyroid hormone. As these potential endocrine-disrupting agents are ubiquitous in low levels, the possible effects on thyroid hormone homeostasis may be pervasive. These agents may unmask or exacerbate existing predispositions to thyroid hormone disorders, such as iodine deficiency, genetic thyroid gland disorders, inherited thyroid hormone receptor or TSH receptor disorders, or pregnancy. In addition, given the profound effects of thyroid hormone in the regulation of the mitochondrial and nuclear genome, endocrine-disrupting agents may pose a particular risk for those with inherited disorders of intermediary metabolism and energy production, including mitochondrial disorders.

Exposure to high levels of PCBs and dioxins has been shown to have direct toxic effects in adults, including severe peripheral neuropathy and encephalopathy (Hsu et al., 1984). In the fetus however, the effects of these agents and their relationship to thyroid hormone in brain development are suspected and may be significant. One of the major effects of thyroid hormone action is in the development of the nervous system. Disorders of fetal thyroid hormone level cause well defined neurologic sequelae which are observed in both animal models and humans (Stein, 1994). Many of the clinical disorders of the brain related to thyroid hormone abnormalities are predicated on abnormalities in the growth and maintenance of neuronal processes and the connections between neurons within and between brain regions (Stein et al., 1991a; Stein, 1994). As such, these disorders represent alterations in cortical functioning and integration. The clinical and neuroanatomic abnormalities, in turn, are the result of selected molecular abnormalities in specific cells in specific brain regions at certain times. The relationship of these endocrine-disrupting agents to abnormal development and maintenance of the nervous system may be mediated by thyroid hormone and possibly modulated by their own independent neurotoxicity. The molecular targets for these agents may be through thyroid hormone regulated nuclear transcription events, as well as extranuclear effects, especially in the mitochondria. Neuronal process growth and maintenance are particularly dependent and sensitive to thyroid hormone during the fetal and neonatal periods. However, the dependency may occur throughout the life cycle.

The purpose of this discussion is to define: (1) the mechanisms by which thyroid hormone regulates the development of the nervous system; (2) typical neurologic and cognitive manifestations of alteration in thyroid hormone using two syndromes of congenital hypothyroidism, human sporadic congenital hypothyroidism (SCH) and an animal model, the hyt/hyt mouse; (3) the molecular and neuroanatomic basis of the adaptive, cognitive, and behavioral abnormalities in animals and humans with thyroid hormone disorders; and (4) the interaction of thyroid hormone on mitochondrial energy generation and function.

This review is focused on thyroid hormone and its molecular, neuroanatomic, and behavioral effects, particularly in the motor system and cerebral cortex. The reader is referred to other extensive reviews of thyroid hormone and fetal brain development (Porterfield and Hendrich, 1991; Stein, 1994), thyroid hormone and iodine deficiency (Escobar et al., 1989; Stein, 1994), neuropsychology of thyroid hormone deficiency (Rovet et al., 1992), neuroanatomic effects of

thyroid hormone (Legrand, 1982–1983), and thyroid hormone in cerebellar and hippocampal development (Rami et al., 1986b; Lauder, 1989). Thyroid hormone and its effects on the brain are presented as a template for understanding potential mechanisms, effects, and future research directions for putative endocrine disruptors.

GENERAL MODEL OF THYROID HORMONE PHYSIOLOGY

The primary signal for synthesis of the thyroid hormones triiodothyronine and thyroxine (T_3, T_4) by the thyroid gland is the production of hypothalamic thyrotropin releasing hormone (TRH). TRH stimulates the production of thyrotropin [thyroid stimulating hormone (TSH)] by the pituitary gland. TSH, in turn, binds to the TSH receptor (TSHr), a G protein linked receptor, in the thyroid gland. This binding begins a cascade of cyclic adenosine monophosphate (cAMP) stimulated events in the thyroid gland which leads to the incorporation of iodine into the thyroglobulin molecule and cleavage of nascent T_3 and particularly T_4 from the complex. These thyroid hormones travel in the bloodstream largely bound to thyroid binding globulin. Smaller amounts are bound to albumin and transthyretin, and a still smaller fraction is free. The free fraction is the biologically relevant moiety. In the peripheral tissue, T_4 is deiodinated to create T_3, the biologically active molecule. Thyroid hormone binds to α or β thyroid hormone receptors and regulates the transcription of specific thyroid responsive genes (Chin, 1992). It may also exert direct extranuclear effects by unknown mechanisms. This system is regulated by the feedback control of T_4 and T_3 on the hypothalamus and pituitary. Modulation of TRH and TSH levels results in strict regulation of thyroid hormone level. Maternal thyroid hormone, transported to the fetus transplacentally, is important for first trimester brain development prior to fetal synthesis, and contributes about 18% of fetal thyroid hormone after autonomous fetal synthesis has begun at 10–12 weeks of gestation (Escobar et al., 1989; Ruiz de Ona et al., 1991).

Disorders of both the hypothalamic-pituitary-thyroid axis and thyroid hormone action lead to a variety of neurologic, neuropathologic, and molecular abnormalities in brain, spinal cord, muscle, and peripheral nerve in the developing and mature human. The timing of the thyroid hormone deficiency and its duration can be used to classify specific thyroid hormone disorders in humans and in animal models of the disorders, e.g. maternal hypothyroidism, endemic cretinism, and sporadic congenital hypothyroidism (SCH) (Escobar et al., 1989; Porterfield and Hendrich, 1991; Stein, 1994). Primary maternal hypothyroidism may affect fetal thyroid hormone levels throughout the pregnancy. Endemic cretinism is due to maternal iodine deficiency. Iodine deficiency produces maternal hypothyroidism as well as reduced fetal synthesis of thyroid hormone, with subsequent fetal hypothyroidism. This may span the entire pregnancy and may be exacerbated by dietary goitrogens which also interfere with thyroid hormone synthesis. In SCH, which occurs in 1/4000 live births in the industrialized world, maternal thyroid hormone levels are normal, but there is deficient synthesis of thyroid hormone by the fetal thyroid gland (Fisher, 1975).

Disorders of thyroid hormone action or responsivity, i.e. resistance to thyroid hormone syndromes (RTH), involve inherited defects in thyroid hormone receptors that interfere with the action of thyroid hormone on the transcriptional activity of specific genes in specific sites of the nervous system and other organs. Secondarily, these patients may develop hyperthyroxinemia which may explain some of the brain and other organ abnormalities (Refetoff et al., 1993).

POSSIBLE ROLES OF POLYCHLORINATED BIPHENYLS IN THE DISRUPTION OF THYROID HORMONE HOMEOSTASIS

Although the production of PCBs in industrialized countries is limited, their hydrophobicity and lipophilicity as well as chemical stability makes them persistent environmental toxicants, with reservoirs in the fat stores of animals. This leads to increasing bioconcentration as one moves up the food chain, and allows facile passage both transplacentally and through lactation. Because of their structural similarities to T_3 and T_4, PCBs may mimic or block the actions of these thyroid hormones and have been shown to decrease both total T_4 and free T_4 in pregnant Wistar rats and, in very high doses, in their fetuses (Morse et al., 1993). The details of possible molecular and biochemical effects of endocrine disruptors on thyroid hormone are presented in other articles herein and are summarized in Figure 1.

Individuals Who May Be at Higher Risk

While all fetuses may be at high risk for developing neurologic and cognitive consequences if exposed to PCBs and dioxins, some may be at even greater risk. These include fetuses from: (1) mothers whose intake of iodine, vitamin A, or selenium is insufficient, whether in areas of malnutrition or endemic cretinism; (2) mothers with undiagnosed hypo- or hyperthyroidism, or with genetic predilection to thyroid disease that is induced by pregnancy; (3) mothers in regions of high exposure to substances that can cause hypothyroidism, i.e. goitrogens from diet or environment (cassava in Zaire or thiocyanates in Lake Michigan fish); (4) mothers with resistance to thyroid hormone syndrome with secondary hyperthyroxinemia; (5) mothers with thyroid stimulating hormone blocking antibodies; (6) mothers on antithyroid treatment for hyperthyroidism; and (7) mothers with marginally compensated metabolic disorders, especially mitochondrial disorders. In addition, fetuses at primary risk include those that will develop sporadic congenital hypothyroidism, resistance to thyroid stimulating hormone, thyroid gland aplasia, or metabolic disorders, particularly mitochondrial disorders. Each of these conditions may have independent effects on the level of thyroid hormone.

GENERAL MODEL OF THYROID HORMONE EFFECTS ON THE NERVOUS SYSTEM

Neurodevelopmental events (neurogenesis, neuronal migration, neuronal differentiation, synaptogenesis, myelination) occur in a temporally and spatially defined sequence in the cerebral cortex (reviewed in Jones, 1981; Jones et al., 1982; Marin-Padilla, 1988; Miller, 1988). Except for myelination and neuronal process maintenance, these events occur principally *in utero*. The development of the human motor system, particularly the premotor cortex, provides a template

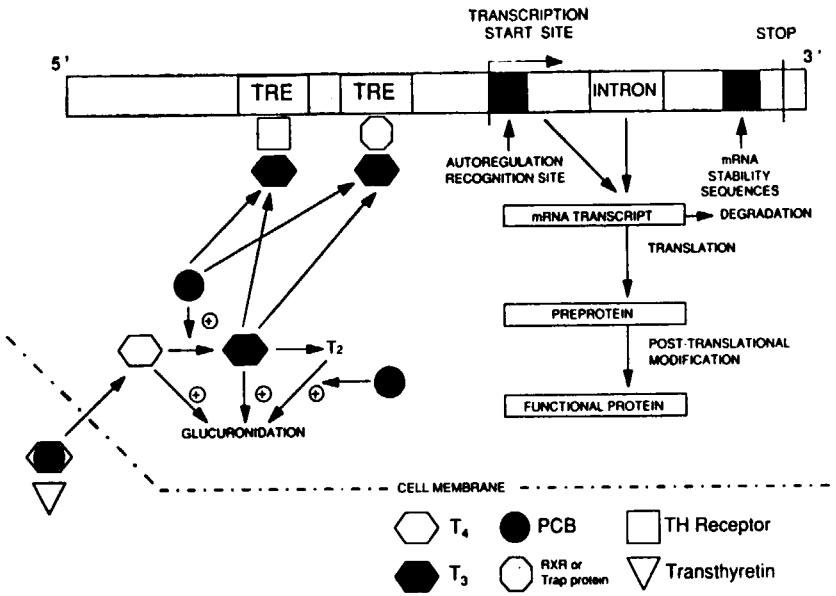

FIGURE 1. Model of thyroid hormone action and the possible effects of PCBs. T_4 circulates primarily bound to thyroid binding globulin, but also to transthyretin, the protein responsible for transport into the brain and likely responsible for transplacental passage. Following passage of T_4 into the cell, it may be deiodinated to reverse T_3 (rT_3) or to T_3. The latter can be inactivated by deiodination to diiodothyronine(T_2). In addition all of the iodothyronines may be further metabolized by glucuronidation, sulfation, deamination or decarboxylation. It has been suggested that PCBs block binding of T_4 to transthyretin and to increase glucuronidation (Morse et al., 1993). T_3 may then bind to a thyroid hormone receptor (TR). It has been suggested that PCBs may compete for TR binding as well (Chin, 1992). According to present models, homodimers of two T_3-TR complexes (or PCB-TR complexes) or heterodimers of one T_3-TR and one T_3-Retinoid X Receptor (RXR) are formed. Only the heterodimer is shown. These complexes then bind to thyroid response elements (TREs) that are organized into two half-sites on specific thyroid hormone responsive genes. The placement of these half-sites is primarily in the 5' flanking region of the thyroid hormone regulated gene but can occur in association with the start site and first exon in the case of some genes, i.e. TSH. These bound complexes interact with the gene to promote or inhibit transcription. Certain genes and their mRNA products may be expressed during certain times of development or maturity, related to transcriptional activation by nuclear proteins, i.e. Pit-1 for growth hormone in the pituitary gland. Retinoic acid and other hormone receptor complexes can also affect transcription by binding or interference with binding at the TREs. Regulation can occur at the transcriptional level, at the level of mRNA stability and degradation, possibly through autoregulation sites or stability sequences, or at the level of translation or post-translational modification, i.e. phosphorylation of neurofilament proteins. (Modified from Stein, 1994.)

for understanding the lifelong effects of thyroid disorders. The pyramidal neurons of the motor cortex, their axonal projections (the corticospinal tracts), and their dendritic processes develop from late in the first trimester throughout gestation, but particularly from 12 weeks to 28 weeks of gestation (Marin-Padilla, 1970; Sidman and Rakic, 1974; Marin-Padilla, 1988). Migration of the layer V pyramidal neurons occurs prior to 12 weeks of gestation. Progressive complexity and interconnection between the dendritic trees of these pyramidal neurons and the development of

the layer IV intracortical neurons and their connections occurs during mid to late gestation. In the layer V neurons, dendritic spines and their connections to other cerebral cortex regions are refined and pruned until six years of age (Conel, 1939–1942). Myelination occurs at least until the second year of life. Process connectivity gives way to process maintenance through adulthood, when neuron loss and process loss occur as old age supervenes.

These neuroanatomic events may be altered by thyroid hormone. In humans and rodents, thyroid hormone can affect neurogenesis, cell migration, axonal and dendritic differentiation, synaptogenesis, myelination, cell death, and neuronal process pruning and maintenance. The age of onset of thyroid hormone disorders temporally corresponds with certain neuroanatomic events and can alter these events. Earlier and more severe insults lead to more severe neuroanatomic and clinical abnormalities. An alteration of thyroid hormone at a specific time of development may affect different neuroanatomical events which are occurring simultaneously. Early effects caused by disorders of thyroid hormone may lead to additional abnormalities, even after the normalization of hormone levels, because of the sequential development of the nervous system.

PCBs and dioxins may exert independent effects on brain development and maintenance as well as influence the level of thyroid hormone in the brain, altering both nuclear and mitochondrial gene expression. The complexity of the individual effects of thyroid hormone add to the difficulty of defining the pathophysiology due to these endocrine-disrupting agents.

NEUROLOGY AND NEUROPSYCHOLOGY OF THYROID HORMONE DISORDERS

Motor System Effects

The neurologic and neuropsychologic abnormalities in fetal, neonatal, juvenile, and adult thyroid hormone disorders are presented in Table 1 and are compared to observations in studies after exposure to PCBs or dioxins at various ages. Clinical manifestations involving the motor system and cerebral cortex throughout the life cycle are well documented in thyroid hormone disorders (reviewed in Stein, 1994). Each of the disorders of fetal thyroid hormone level demonstrates neurologic and neuropsychologic manifestations that relate to intrauterine fetal hypothyroidism of differing timing, duration, and severity, suggesting that the motor system and cerebral cortex are major targets for thyroid hormone throughout normal development (Figure 2).

In subjects with endemic cretinism, a specific motor syndrome is universally noted. This motor syndrome involves proximal spasticity, altered reflexes, pathological reflexes, and hemiparesis (Delong, 1989; Halpern et al., 1991). The syndrome also commonly includes axial rigidity, flexion dystonia, mask-like facies, and gait abnormalities, suggesting basal ganglia involvement. Slowness in initiation of action and apraxia are also noted. The severity of motor disabilities in children born to mothers with endemic cretinism is correlated with maternal T_4 levels (reviewed in Stein, 1994).

Table 1
Neurological and Electrophysical Abnormalities in Thyroid Hormone Syndromes

	Adult Hypot. %	Adult Hypert. %	Cong. HT (RX>3 months)	Cong. HT (RX<1month)	RTH	Neonatal Hypert.	Endemic Cretinism	Mat. HT.	Perinatal PCB/Dioxins	Adult PCB toxic exposure
Mental Status Abnormalities										
Level of conciousness/orientation										-
Drowsiness	22	-	+/-	+/-					?	-
Vigilance	31	+	+/-	+/-	+					?
Seizures		+	+	+	+/-	+	+			
Sluggishness				+/-						
Coma	2				+/-					
Thinking disorder										
Psychosis	3 - 36	+	-	-	+/-				+/-	?
Dementia/Memory dis.	30 - 65	-	+	+					?+	?
Mental retardation			+ to +++	+/-	+/-	+	+ to +++	+	?+	
↓ Verbal IQ		+/-	+	+/-	+/-				?+	
↓ Performance IQ			+	+/-	+/-				?	
Speech abnormalities										
Aphasia	-	-	+	+/-	+/-					
Dysarthia	-	-	+	+/-	+/-					
Cortical integration abnormalities										
Visuospatial dysfunction				+/-	+/-		+		?	?
Visuomotor dysfunction				+/-	+/-		+			
Reading abnormality/Dyslexia		+/-		+/-	+/-					
Spelling abnormality				+/-	+/-					
Math abnormality				+/-	+/-					
Disorder of planning	+	+		+/-	+/-					
Gen. learning disability			+	+/-	+/-	+		+		
Right-left disorientation					+/-					
Other disorders										
Attention Deficit Disorder (ADD)			+	+	+					
Mood or depression	40	+	?	?	+/-					
Anxiety disorder	13	99	+/-	+/-	+/-					
Emotional liability	15	+	+	+	+/-					
Psychosis										
Insomnia/Sleep disorder	9 - 30	+	?	?	+/-					
Hyperactivity		+	+	+/-	+/-					
Behavioral disorder			+	+	+					
Apathy	40	+	+	+	+					
Intracranial hypertension	+/-	+/-			+/-	+				
Microcephaly			+		+/-	+	+		+/-	
Hydrocephalus					+/-	+				
Basal Ganglia Dysfunction										
Rigidity							+		?	
Tremor		+	+	+	+					
Chorea		+								
Masked facies	82									
Cranial Nerve/Brainstem Abnor.										?
↓ Visual Activity/optic neuropathy	45				+/-				?	
Oculomotor palsy			+/-	+/-	+/-					
Eye movement disorder			+/-	+/-	+/-					
Diplopia	5		+	+	+/-					
Nystagmus					+/-					
7th Nerve palsy			-		+/-					
Hearing loss	3 - 53		+	+/-	++		+	?	?	
Vertigo	12									
Swallowing dysfunction					+/-					
12th Nerve palsy					+/-					
Motor Abnormalities										?
Gait	?		Clumsy	Clumsy			Distinct			
Hemiparesis	?	?	+	+			+	+	+/-	?
Anterior horn cell disease							+		?+	
Cerebellar abnormalities										
Truncal ataxia	27		+	+/-			-			
Appendicular ataxia	7 - 52		?	?	-					

Neurological and Electrophysical Abnormalities in Thyroid Hormone Syndromes (continued)

	Adult Hypot. %	Adult Hypert. %	Cong. HT (RX>3 months)	Cong. HT (RX<1month)	RTH	Neonatal Hypert.	Endemic Cretinism	Mat. HT.	Prenatal PCB/Dioxins	Adult PCB toxic exp.
Motor Abnormalities (cont'd)										
Reflexes										
Areflexia	10									
Hyporeflexia	44									
Tone										
Spasticity	?	+	+				+++	+		?
Hyperreflexia	?	+	+				+++	+		
Rigidity							+++	-		
Hypotonia	?	+	+	+	-		+		+/-	
Peripheral Nerve Abnormalities										
Small fiber sensory neuropathy	13	+								
Large fiber neuropathy	10	+								+
Carpel tunnel syndrome	2									+/-
Paraesthesias	13									
Median nerve abnormalities	23									
Motor neuropathy										+/-
Distal combined neuropathy	+	+								
Segmental demyelination/axonal loss on nerve biopsy	+	+								
Muscle Abnormalities										
Hypertrophy	+									
Proximal weakness	34	50 - 80	+	+/-	+/-	+/-	+		?	?
Atrophy							+			
Cramps	6 - 10	7 - 46								
Myopathy	1		?							
Pain	50									
Myasthenia gravis	-	1								
Periodic paralysis	-	2 - 8								
EMG polyphasia	6									
EMG CMAP (Abnormal)	6									+
Conduction velocity (Abnormal)	+	+		+						
Myopathic EMG	+	95								
Abnl. muscle biopsy	68 - 77	+	?							
Pseudomyotonia	+									

Adult Hypot. = adult hypothyroidism; Adult Hypert. = adult hyperthyroidism; Cong. HT (RX > 3 Months) = late treated congenital hypothyroidism prior to screening programs; Cong. HT (RX < 1 Month) = congenital hypothyroidism treated prior to 1 month-of-age; RTH = homozygotes and heterozygotes with resistance to thyroid hormone syndrome compared to controls (Stein et al., 1991b) in Kindred S (Ono et al., 1991) and other Kindreds (Refetoff et al., 1993; Hauser et al., 1993); Neonatal Hypert. = Neonatal Graves disease; Mat. HT. = primary maternal hypothyroidism or hyperthyroidism during pregnancy. The effects of endemic cretinism and maternal thyroid disease reflect assessment of the progeny of pregnancies complicated by these disorders. + = present degree of abnormality; - = not present; +/- = present in some patients with the indicated disorder; ? = unknown or possibly; numbers = the % occurrence of this sign/symptom in large population studies. PCB and Dioxin data are drawn from Fein et al., 1984; Chia and Chu, 1985; Jacobson et al., 1985; Rogan et al., 1986; Koopman-Esseboom et al., 1994 & 1996; Huisman et al., 1995; and Lonky et al., 1996. [Reviewed and data cited in Stein et al., 1994.] Modified from Stein, 1994.

Similarly, in human sporadic congenital hypothyroidism, untreated patients or patients treated after 1–3 months of age demonstrate motor signs suggestive of cerebral palsy, including gross and fine motor clumsiness, spasticity, poor coordination, basal ganglia movement disorders, and cerebellar ataxia (MacFaul et al., 1978; Wolter et al., 1979). Even patients treated within the first 3 months after birth showed slowed motor performance, pyramidal signs, and overt spasticity.

Studies of SCH patients treated before one month of age have produced conflicting results. Gross motor examination of the patients in the New England Congenital Hypothyroidism Collaborative and neurologic and neuroradiologic examination with magnetic resonance imaging of a small group of Quebec patients were normal (New England Congenital Hypothyroidism Collaborative, 1984, 1985, 1990; Dussault et al., 1993). On the other hand, some patients with

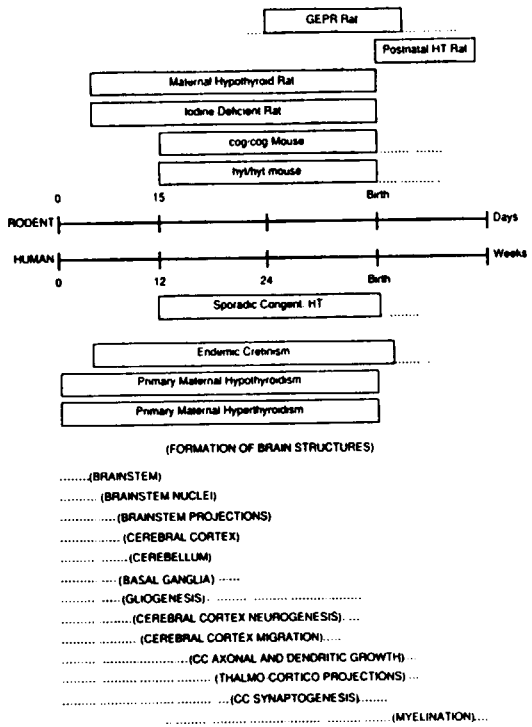

FIGURE 2. Timing of disorders of thyroid hormone level and their relation to age and neuroanatomic development in humans and animal models. During the first trimester for humans and the first 15 days for rodents, the fetus is dependent entirely on transplacental transport of maternal thyroid hormone. The fetal thyroid gland starts to synthesize thyroid hormone at 10–12 weeks in the human and 15 days in the rodent. After that time, the fetus principally uses its own thyroid hormone, but some thyroid hormone is still transported from the mother. Disorders of thyroid hormone level include primary maternal hypothyroidism, endemic cretinism, and sporadic congenital hypothyroidism. Maternal hypothyroidism can only influence fetal brain development *in utero*. Endemic cretinism, related to iodine deficiency, can influence fetal brain development through the duration of the pregnancy and persist through life if the iodine problems within a society or country are not corrected. Sporadic congenital hypothyroidism (SCH) is purely a failure of autonomous synthesis of thyroid hormone of the fetal thyroid gland that affects the fetal brain even though it receives normal maternal thyroid hormone levels. Exposure to PCBs and dioxins, which may reduce thyroid hormone, can influence development both *in utero* and postnatally, through transplacental and lactational passage. The duration and timing of the exposure may determine the similarity to endemic cretinism with maternal and postnatal hypothyroidism, maternal hypothyroidism, or SCH. Disorders of thyroid hormone and those related to endocrine disruptors may occur throughout life from the postnatal period to adulthood. The major brain structures, such as brainstem and cerebral cortex (cc), are usually formed before about 8 weeks of gestation. Following neurogenesis, migration, and differentiation of neurons, synaptogenesis, selective cell death, and myelination occur. Myelination can occur for prolonged periods postnatally; refinement and pruning of neuronal processes both of axons and dendrites can occur up to at least six years of age. The maintenance and plasticity of neuronal process efficacy and dendritic spines occur throughout the life cycle. All events are sensitive to changes in thyroid hormone level. The hyt/hyt mouse is useful for evaluation of the effects of thyroid hormone throughout the life cycle, but is particularly relevant for the period from 15 days post conception to birth, a time of synthesis of critical molecules and process outgrowth and elongation, particularly of pyramidal neurons in the sensorimotor cortex and hippocampus. (Reproduced with permission from Stein, Cognizant Communications Corporation, 1994). HT=hypothyroid; GEPR=the hypothyroid inbred rat with seizures.

early treatment have fine and gross motor dysfunction, coordination and balance abnormalities, and reduced speed/dexterity (Rochiccioli et al., 1989; Fuggle et al., 1991; Kooistra et al., 1994). Coordination and fine motor function were significantly impaired in grade repeaters with SCH who also had a higher incidence of athyreosis (Rochiccioli et al., 1992). The motor abnormalities were associated with IQ reductions and more severe fetal hypothyroidism. These motor system abnormalities provide the strongest evidence for an irreversible effect on the fetal nervous system.

The clinical findings in these disorders can be related to abnormalities in motor regions of the brain and spinal cord. These include the corticospinal tracts, parietal regions involved in motor behavior, premotor cortex, primary motor area, and the supplementary motor area. In addition, the medial and medial lateral precentral gyrus, the basal ganglia and cortical pathways appear to be involved. In some patients, dysfunction at the level of the anterior horn cell is seen. Findings in human autopsies include reduced numbers of pyramidal and Betz neurons in layer V of the cerebral cortex, abnormalities in pyramidal neuron axonal and dendritic development (Marinesco, 1924; Lotmar, 1928; Jia-Liu et al., 1989), and diminished size of the pyramidal tracts in the cerebral cortex and brainstem (Rosman, 1975). The pathological lesions seen in disorders of fetal hypothyroidism are similar to those seen in cerebral palsy. Disorders of neurogenesis and intracortical interconnections of neuronal processes lead to reduction in head circumference. In severe endemic cretinism and fetal or neonatal hypothyroidism, microcephaly is frequently seen (Boyages et al., 1988).

Cognitive Effects

Mental retardation and learning disability are particularly common in thyroid hormone disorders. The cerebral cortex, including the frontal and parietal lobes, and the hippocampus are targets for thyroid hormone, and clinical evidence of dysfunction can be related to these specific brain regions. In the past, hypothyroidism was a common cause of moderate to severe mental retardation (IQs < 70)(Smith et al., 1957), because in many cases adequate T_4 therapy was started after 3–6 months of age. In studies with T_4 treatment after 2–3 months of age, speech defects (including mild expressive aphasia and articulation disorders), hyperactivity with poor attention (MacFaul et al., 1978), and perseveration (Money, 1956) were frequently observed. In the industrialized world, mandatory neonatal blood screening programs and treatment with thyroxine (T_4) have effectively eliminated mental retardation (New England Congenital Hypothyroidism Collaborative, 1984, 1985, 1990; Rovet, 1989; Fuggle et al., 1991; Rovet et al., 1992; Kooistra et al., 1994), but reductions in IQ have still been observed in certain cases (Rovet et al., 1992; Dussault et al., 1993). The pre-screening studies illustrate that delays in diagnosis and treatment of SCH result in significant cognitive, behavioral, and neurologic dysfunction that are irreversible. The severity of the disorders was correlated to the length of time after birth before T_4 therapy was initiated, suggesting that normal T_4 levels after birth are essential for preventing mental retardation.

In a series of longitudinal studies using a broad-based battery of neuropsychologic tests, the New England Collaborative Group (1984, 1985, 1990) demonstrated that early treatment of SCH patients with thyroxine prevented decrements in IQ, learning disabilities, and abnormalities in school achievement. Despite these observations, several excellent longitudinal studies in many countries have found a variety of abnormalities in patients with SCH. These included small but

significant reductions in performance and verbal IQ (reviewed in Fuggle et al., 1991), specific learning disabilities and attention deficit disorder (Rovet, 1989; Glorieux, 1989; Rochiccioli et al., 1992; Rovet et al., 1992), problems with school achievement (Rochiccioli et al., 1992), fine and gross motor abnormalities (Rochiccioli et al., 1989; Fuggle et al., 1991; Kooistra et al., 1994), hearing deficits, and abnormalities in visual and somatosensory evoked responses (Laureau et al., 1986). These effects were particularly noted in those patients with evidence of severe fetal hypothyroidism manifested by delayed bone age and T_4 levels < 0.5 µg/dL. In fact, the prognosis varied significantly between patients with severe fetal hypothyroidism and those with moderate fetal hypothyroidism. Glorieux et al. (1992) noted significantly lower verbal IQ, non-verbal IQ, and global IQ in those with severe congenital hypothyroidism compared to those with moderate congenital hypothyroidism. In children with endemic cretinism, iodine replacement programs have significantly improved the incidence of mental retardation, but reduction in IQ and motor abnormalities may still be observed (Boyages et al., 1988).

The adult or child with spontaneous onset of hypothyroidism may manifest alterations in consciousness and a variety of psychiatric disorders, such as lethargy dementia, depression, psychosis, and vigilance disorders (Swanson et al., 1981; Joffe and Sokolov, 1994). These reflect abnormalities in the prefrontal cortex, cortical interconnections, the limbic system, and visual and auditory corticies.

NEUROLOGY AND NEUROPSYCHOLOGY OF PUTATIVE ENDOCRINE DISRUPTOR TOXICITY

Prenatal Exposure to PCBs and Dioxins

In humans, the effects of prenatal and early postnatal exposure to potential thyroid-disrupting substances have been evaluated in the Lake Ontario region of New York (Lonky et al., 1996), the Lake Michigan region (Fein et al., 1984; Jacobson et al., 1985, 1990a,b; Jacobson and Jacobson, 1996), in North Carolina (Rogan et al., 1986; Gladen et al., 1988), in the Netherlands (Koopman-Esseboom et al., 1994, 1996; Brouwer et al., 1995; Huisman et al., 1995), and in Taiwan (Hsu et al., 1984; Rogan et al., 1988). These studies evaluated neonatal and long-term childhood development using different instruments for assessment of neurologic, somatic, and neuropsychologic development.

In North Carolina, using the Brazelton assessment tools (Neonatal Behavioral Assessment Scale), Rogan et al. (1986) demonstrated that increasing PCB levels in neonates were related to hypotonia and hyporeflexia, suggesting anterior horn cell dysfunction. Using similar assessment tools, Lonky et al. (1996) studied infants delivered to mothers who consumed fish from Lake Ontario. They found that these infants had more abnormal reflexes, abnormal autonomic performance (more startles and tremors), and reduced habituation with persistent overreaction to stimuli. Poorly developed orientation to auditory and visual stimuli was also seen. In addition, there appeared to be a dose-response relationship: infants of mothers who were higher consumers of fish (with presumed ingestion of higher levels of PCBs, dioxins, dieldrin, lindane, cadmium, and mercury) showed a greater number of abnormal reflexes compared to infants of mothers who were lower or non-fish consumers. Infants of lower consumers of fish also differed from those of non-fish

consumers in reflexes and habituation, suggesting that even low level exposure was important. Similarly, Jacobson et al. (1985), in the Lake Michigan area, demonstrated that maternal consumption of PCB-contaminated fish was related to neonatal motor immaturity, state lability, and diminished reflexes.

Perinatal Exposure to PCBs, Dioxins and Furans

Perinatal exposure to PCBs, dioxins, and furans from placenta and via breast milk has been evaluated in the Netherlands in a semi-urban area and a highly industrialized area (Koopman-Esseboom et al., 1994, 1996). Prenatal exposure to four PCB congeners, was not related to alterations in a detailed neonatal neurologic examination (Huisman et al., 1995). On the other hand, postnatal exposure to 26 PCBs, dioxins, and furans was related to reduced neonatal neurologic optimality (Huisman et al., 1995). [Neurologic optimality involves semi-quantitative assessments of deep tendon reflexes, grasp responses, and a series of indices of postural tone (Prechtl, 1977, 1980; Touwen et al., 1980) that are totaled to develop a neurologic optimality score.] Specifically, the highest PCB exposed group had reduced optimality and a higher prevalence of hypotonia. Severe neurologic abnormality was not seen.

Humans exposed inadvertently to large amounts of PCBs, as in the cases of Taiwanese oil contamination (Hsu et al., 1984; Rogan et al., 1988), have shown some concerning effects as well. Infants born to mothers exposed to this oil demonstrated intrauterine growth delay and persistent delayed growth in early childhood, and in longitudinal studies, scored significantly lower on intelligence tests than age matched controls (Rogan et al., 1987; Chen et al., 1992).

These studies indicate that the fetal motor system may be a target for endocrine-disrupting agents, but do not substantiate a thyroid hormone mediated effect. The findings related to fish consumption are difficult to interpret because: (1) multiple toxicants may be present; (2) these toxicants may not have specific effects on fetal thyroid hormone; and (3) multiple toxicants may produce effects that previously have been attributed to single toxicants.

Another problem common to studies of neonates is that early neonatal examinations may not predict later development. Of the tools used, the neonatal neurologic evaluation of Touwen et al. (1980) and Prechtl (1977, 1980) may be the most reliable. Later neurologic examination, at seven months, has been reliably used to diagnose motor dysfunction classified as cerebral palsy in other contexts (Nelson and Ellenberg, 1986).

In contrast, neonatal head circumference may have more predictive value. In the Lake Michigan study, detectable PCBs in cord blood predicted reduction in head circumference (Fein et al., 1984). This may be related to developmental effects on the fetal brain, similar to those seen in fetal thyroid disorders. Nevertheless, this reduction of head circumference was not seen in other studies (Rogan et al., 1986; Dar et al., 1992). However, the relative concentrations of specific toxicants among the studies are not clear.

Extending these studies to later childhood, several psychological studies have demonstrated lower IQs and abnormalities in memory and vigilance related to perinatal and prenatal exposure to endocrine disruptors (Hsu et al., 1984; Jacobson et al., 1985; Gladen et al., 1988; Jacobson et al., 1990; Chen et al., 1992; Chen and Hsu, 1994; Jacobson and Jacobson, 1996; Koopman-Esseboom et al., 1996). Visual evoked responses may also be abnormal in these patients (Chen et al., 1992).

Adult Exposure to PCBs and Dioxins

Neurologic and electrophysiologic studies of patients exposed to acute ingestion of PCBs in cooking oil demonstrated a clinical peripheral sensory neuropathy, as well as reduction in nerve conduction velocity (Chia and Chu, 1985). This study reported normal electroencephalograms and evoked potentials in these subjects. Although headaches and dizziness were reported in increased frequency, no detailed neurologic or neuropsychologic studies have been done. The effects appear to be specific to peripheral nerve and cannot be attributed to an effect on thyroid hormone. Most likely they are the direct toxic effects of PCBs.

In summary, the human studies show motor, tone, and reflexive abnormalities in the neonatal period with later psychomotor delay and significant reduction in IQ that relate to prenatal and perinatal exposure to putative endocrine disruptors. The levels of PCBs, the specific agents, and the effects vary among the studies, as do the testing instruments used. Although some abnormalities are similar to those seen in thyroid disease (hypotonia, reflex abnormalities, poor growth, and decreased head circumference), the specificity of these effects to thyroid hormone defects cannot be determined from the data.

ANIMAL MODELS OF HUMAN THYROID HORMONE DISORDERS

An understanding of thyroid hormone disorders comes from molecular, neuroanatomic, neuroendocrinologic, and behavioral analysis of animal models. Specific animal models can be related to specific human thyroid hormone conditions (Figure 2 and 3). These animal models are advantageous for a number of reasons: (1) The types, sites, and patterns of the neurological, neuropathologic, and behavioral abnormalities are similar in rodent and human thyroid hormone disorders (reviewed in Stein et al., 1991a). (2) As in humans, rodents follow a fixed progressive development of motor and adaptive behavior that begins *in utero* and continues postnatally (Almli and Fisher, 1977; Anthony, 1990). (3) These models can be used to identify the molecular and neuroanatomic targets for thyroid hormone and its second messengers in the normal brain. (4) These animal models may be useful for furthering our understanding of endocrine disruptors.

PCB Exposure as a Model of Maternal Hypothyroidism

We have compared the fetuses from rodent models of hypothyroidism and endemic cretinism to those of dams exposed to PCBs. In Table 2, the brain levels of thyroid hormone per gram of brain tissue are shown for these models. Despite differing methods of measurement, these models exhibit reduced T_4 and T_3 in fetal brains. Morse et al. (1996a) exposed pregnant Wistar rats to two different doses (5 mg/kg/d and 25 mg/kg/d) of Aroclor 1254, a PCB analog, and measured T_4 and T_3 in brains of the offspring. Fetal (GD 20) and postnatal day 21 brains were assayed for levels of thyroid hormone. There appeared to be an inverse dose-response relationship, with the highest exposure resulting in thyroid hormone levels approximating those seen in conditions of maternal thyroid ablation. By postnatal day 21, the reduction in T_4 (1.3 mg/g) persisted but T_3

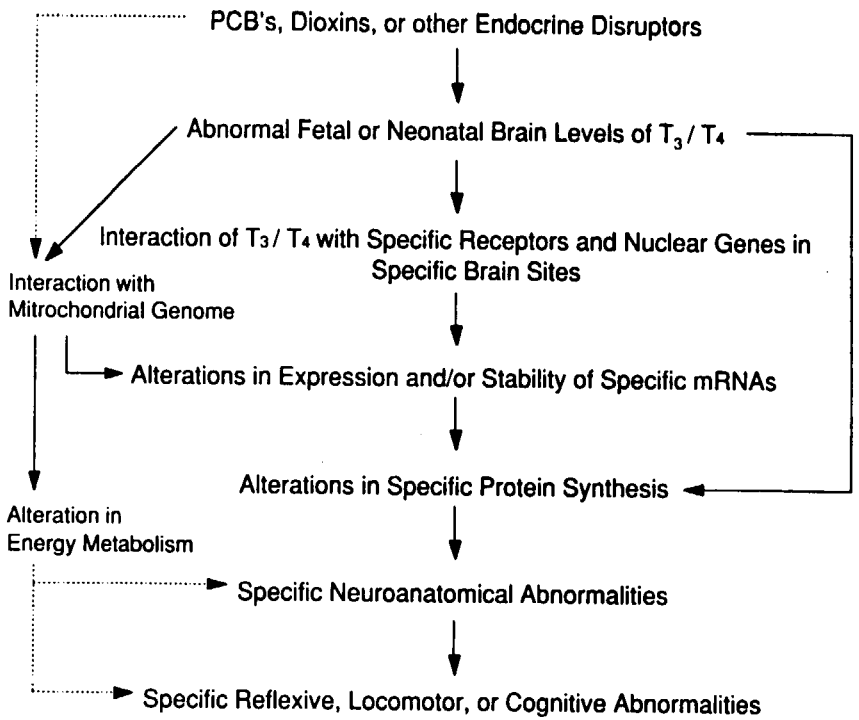

FIGURE 3. Model of thyroid hormone action and brain development: Mechanisms for production of anatomical and behavioral abnormalities. Alterations in fetal serum and brain T_3/T_4 levels, following interaction with thyroid hormone receptors in specific brain sites or with specific proteins or transitional apparatus, may lead to alterations in expression or stability of some mRNAs or proteins. These mRNA or protein abnormalities occurring during certain times in gestation or in the neonatal period may lead to neuroanatomic abnormalities. The type of neuroanatomic abnormalities depend on the normal events that are sequentially occurring at the time of the hypothyroidism. These neuroanatomic abnormalities contribute to some of the deficits in reflexive, locomotor, or adaptive behavior that are observed in hypothyroidism. PCBs, dioxins, or other endocrine disruptors may act to lower thyroid hormone levels and lead to similar hypothyroid related effects. Alternatively, disorders of thyroid hormone level, or endocrine disruptors independently, may interact with the mitochondrial genome to alter the expression of specific mRNAs or alter cellular energy metabolism. The latter may represent another mechanism by which disorders of thyroid hormone level and endocrine disruptors may create neuroanatomic and behavioral abnormalities. Parts of this model for thyroid hormone disorders have been tested and validated with the hyt/hyt mouse (Stein, 1994). (Modified from Stein et al., 1991a)

TABLE 2. Brain Thyroid Hormone Levels in Rodent Thyroid Disorders: Aroclor 1254 Compared to Models of Maternal Hypothyroidism

	Control ng/g		Hypothyroid ng/g		Method/Species	Reference
Day	T_4	T_3	T_4	T_3		
GD 13	1.7	1.2	0.8	0.6	I^{131}/Sprague Dawley	Porterfield and Hendrich, 1992
GD 16	1.7	1.4	0.9	0.8	as above	as above
GD 18	.114	.068	.046	.048	Thyroidectomized/Wistar	Ruiz de Ona et al., 1991
GD 21	2.1	0.8	0.2	0.1	MMI/Wistar	Pedraza et al., 1996
GD 20	1.5	1.1	0.3	0.2	MMI/Unspecified Rats	Escobar et al., 1989
GD 20	1.8	0.5	0.2	0.3	Aroclor 1254/Wistar	Morse et al., 1996a
GD 21	1.5	1.1	0.2	0.2	LID/Wistar	Obregon et al., 1991
GD 22	10	9	10	7	Thyroidectomized/ Unspecified Rats	Porterfield and Hendrich, 1991
PND 21	2.0	3.3	1.3	3.5	Aroclor 1254/Wistar	Morse et al., 1996a

I^{131}, radioactive iodine thyroid ablation; MMI, methimazole thyroid ablation; LID, lithium induced hypothyroidism.

normalized (3.5 mg/g). Hence, during the fetal period, Aroclor 1254 appeared to cause brain hypothyroidism; however, this effect was transient, This may be due to direct effects of Aroclor on Type II 5' deiodinase, transthyretin, or glucuronidation and hence the effect on the free levels of these hormones is difficult to determine. Some of the behavioral and anatomic abnormalities observed in fetally exposed animals may also be observed in animals with endemic cretinism or offspring of hypothyroid mothers.

The hyt/hyt Mouse: A Model of Human SCH and Endocrine Disruptor Effects

We have studied a reliable animal model of sporadic congenital hypothyroidism, the hyt/hyt mouse, which can be used to elucidate not only the effect of thyroid hormone on brain development, but also the effect of endocrine disruptors on thyroid hormone and the brain. This model is specifically relevant to SCH because, as with humans, the timing of onset of the hypothyroidism corresponds with the beginning of autonomous fetal thyroid hormone secretion. The severity of the fetal hypothyroidism in the hyt/hyt mouse is similar to patients with SCH and severe fetal hypothyroidism who are more likely to demonstrate learning disabilities despite early T_4 treatment. Furthermore, the defect is lifelong and, as such, permits study of the effects of hypothyroidism throughout the life cycle. Our work with this animal model has recently been extensively reviewed (Biesiada et al., 1996).

The hyt/hyt thyroid gland makes a reduced amount of thyroid hormone as well as all other components required for normal thyroid hormone synthesis (Table 3). Although the gland is hypoplastic, all essential anatomic structures are present. Compared to its euthyroid hyt/+ littermate mice and progenitor strain BALB/cBY +/+ mice, the hyt/hyt mouse has a 5–10-fold reduction in serum thyroxine, a 16-fold decrease in triiodothyronine, and a 100-fold elevation in TSH-like activity (Adams et al., 1989; Stein and Adams, 1989; Stein et al., 1989). This mouse has a biologically active TSH molecule (Stein et al., 1991b), and the response of hyt/hyt glands *in vitro*

TABLE 3. Characterization of the hyt/hyt Mouse

Index/Observation	Level/Presence	Reference
Thyroid Function		
T_4[a,b]	↓(5-10X)[c]	Stein et al., 1989
Intrathyroid T_4	↓	Beamer and Cresswell, 1982
T_3[a]	↓(16X)[c]	Stein et al., 1989
TSH[c]	↑(100X)[c]	Stein et al., 1989
TRH	↑[d]	Noguchi et al., 1986
Thyroid gland hypoplasia[a,b]	+(5X)	Stein et al., 1989
Varied follicular diameter[a,b]	+	Stein et al., 1989
Small follicles[a,b]	+	Stein et al., 1989
Reduced follicular cytoplasm[a,b]	+	Stein et al., 1989
Follicular cell number[a,b]	↑(2X)	Stein et al., 1991c; Biesiada et al., 1996
Size and number of microvilli[a,b]	↓	Shanklin and Stein, 1988; Biesiada et al., 1996
Amount of follicular endoplasmic reticulum[a,b]	↓	Biesiada et al., 1996
Amount of colloid[a,b]	↓	Stein et al.,1989
Nuclear ARC ratio (Measure of share of nucleus and cytoplasm per follicular cell)[a,b]	↑	Shanklin and Stein, 1988; Biesiada et al., 1996
Bioactive TSH[c]	+	Stein et al., 1991c
Iodinated thyroglobulin	+	Stein et al., 1989
Mono/diiodotyrosines	+	Stein and Taurog, unpublished
Thyroglobulin processing	Normal	Stein et al., 1989
Thyroid gland iodine uptake	↓	Beamer and Cresswell, 1982
Thyroid gland cAMP[a,b]	↓[c]	Stein et al., 1991c
Thyroid gland response to TSH[a,b]	Minimal	Stein et al., 1991c
Thyroid gland Gs function[a,b]	Normal	Stein et al., 1991c
Thyroid gland adenyl cyclase[a,b]	Normal	Stein et al., 1991c
Thyroid gland TPO mRNA[a,b]	↓(3X)[c]	Stein et al., 1991c
Thyroid gland thyroglobulin mRNA[a,b]	↓(4z16X)[c]	Stein et al., 1991c
Size and level of thyroid gland TSH receptor mRNA[c]	Normal	Stein et al., 1991c
Replacement of Pro with Leu in TMD IV of TSHr	+	Stein et al., 1994
Binding of TSH to TSHr	Absent	Stein et al., 1994
Processed and mature TSHr protein	Present	Gu et al., 1995
Expression of TSHr on cell surface	Present	Gu et al., 1995; Biesiada et al., 1996
Somatic/Neurobiological		
Day of eye opening[a,d]	Delayed	Adams et al., 1989
Day of ear raising[a,d]	Delayed	Adams et al., 1989
Reflexive behavior[a,d]	Delayed	Adams et al., 1989
Corticospinal tract-mediated behaviors	Delayed	Biesiada et al., 1996; and this paper
Locomotive/cognitive function[a,d] (see Table 4)	Abnormal	Stein et al., 1991c; Anthony et al., 1993; Biesiada et al., 1996
Cerebral cortex/hippocampus[a,d]	Abnormal	Stein et al., 1991c
Fetal cerebral cortex mRNA reduction (ECF, tubulin isoforms)[a,d]	+	Stein et al., 1989, 1991c; Biesiada et al., 1996
Fetal cerebral cortex protein reduction (tubulin isoforms)[a,d]	+	Stein et al., 1989; Biesiada et al., 1996
SCa and SCb axonal transport	↓	Stein et al., 1991a, 1991c
Axonal levels of tubulin/calmodulin[a,d]	↓/↑	Stein et al., 1991a, 1991c
Axonal transport of tubulin, actin, spectrin, neurofilament[a,d]	↓	Stein et al., 1991a, 1991c

+ = present; ↓ = decreased; ↑ = increased; [c]TSH regulated event; [b]Abnormal in fetal and neonatal thyroid; [c]Statistically significant changes; [d]T_4 responsive; Gs = G stimulating protein; SCa = slow component A axonal transport; SCb = slow component B axonal transport; TMD = transmembrane domain; TPO = thyroid peroxidase; TSHr = TSH receptor. Modified from Biesiada et al., 1996.

to TSH is significantly reduced compared to control animals. The basal level of cAMP in the hyt/hyt thyroid gland is reduced compared to the hyt/+ mouse, but cAMP production is equivalent when the hyt/hyt and control thyroid glands are exposed to other stimuli (Stein et al., 1991b). Despite a 100-fold increase in circulating TSH, the gland is hypoplastic, consistent with TSH hyporesponsiveness of the thyroid gland. In humans, current estimates of the prevalence of SCH due to TSH hyporesponsiveness range from 1/10 000–1/80 000 live births.[1] Given that the risk of SCH is 1/4000, this could mean that up to 40% of SCH is due to TSH hyporesponsiveness. In fact, mutations in the TSH receptor have now been described (Kopp et al., 1995; Sunthornthepvarskul et al., 1995), including a mutation in transmembrane domain IV, close to the mutation seen in hyt/hyt mice[2] (see below).

Behavioral impairment in the hyt/hyt mouse. The behavioral studies of the hyt/hyt mouse have been designed to measure corticospinal tract function, early reflexes, and locomotor and adaptive behavior in neonatal through young adult animals (Altman and Sudarshan, 1975; Almli and Fisher, 1977; Donatelle, 1977). All of the behavioral abnormalities in hyt/hyt mice are shown in Table 4. Of particular note, delays in forepaw and hindpaw grasping were observed in hyt/hyt mice, but not their euthyroid littermates or +/+ control mice (Adams et al., 1997). These delays resolved over time. Despite eventual attainment of normal performance, the delays in grasping suggest a slower development of the corticospinal neurons and of rostral-caudal motor function maturation that may underlie later abnormalities in more complex motor behavior.

Hyt/hyt mice also demonstrated reductions in locomotor activity compared to euthyroid mice at days 21, 40, and 120 (Adams et al., 1989) and increases at 14 days. This may have consequences for later learned behavior. Simple learned motor behavior was evaluated by the swim escape paradigm (Adams et al., 1989), and more complex learned behavior was evaluated by the Morris water maze (Anthony et al., 1993). Hyt/hyt mice showed no ability to learn swim escape or the distal Morris maze but showed some improvement in response time for the proximal Morris maze (Anthony et al., 1993). Spatial learning, ascertained by the distal Morris water maze testing, is related to the anatomic integrity of the hippocampus and visual motor integration. The reduced size of the hippocampus in the hyt/hyt mouse may be related to reduced numbers and process growth of hippocampal neurons (Rabie et al., 1979; Rami et al., 1986a,b). Similarly, permanent impairments in cognitive and complex motor functions are seen in untreated and late-treated human SCH (MacFaul et al., 1978; Wolter et al., 1979; Klein, 1985) and in some patients with high level PCB exposure *in utero* (Chen et al., 1992; Chen and Hsu, 1994).

Neuropathologic studies in the hyt/hyt mouse and other hypothyroid rodent models. Hypothyroidism leads to reductions in the ability to initiate, elongate, and maintain neuronal processes. It also results in reductions in the number of process branches and dendritic spines, changes in the distribution of dendritic spines, and alterations in synaptogenesis and synaptic maintenance. Alterations in neuronal ionic pumps and reductions in myelination resulting in impaired axonal conduction have also been observed (Marinesco, 1924; Rosman, 1975; Legrand, 1982–1983; Stein et al., 1991a; reviewed in Stein, 1994). Thus, alterations in thyroid hormone

1. Refetoff, S. (1996). Personal communication. Presentation at the Conference of the American Thyroid Association, November, San Diego, CA.
2. Vassart, G. (1996). Personal communication. Presentation at the Conference of the American Thyroid Association, November, San Diego, CA.

TABLE 4. Behavioral Abnormalities in the Hypothyroid and Hyperthyroid Rodent and Hypothyroid hyt/hyt Mouse and Endocrine Disruptor Exposed Animals

Type of Behavior	Specific Defect	Days of Age	Result	Thyroid Disorder
REFLEXIVE	Forelimb Placement	1–5 days	Delayed	hyt/hyt
	Hindlimb Placement	1–5	Delayed	hyt/hyt
	Olfactory Orientation	3–7	Delayed	hyt/hyt
	Air Righting	14–18	Accelerated	NHyp
	Cliff Avoidance	4–14	Delayed	hyt/hyt
	Negative Geotaxis	4–14	Delayed	hyt/hyt
	Forepaw Grasping	5–10	Delayed	hyt/hyt
	Forepaw Hanging	5–10	Delayed	hyt/hyt
	Hindpaw Hanging	7–11	Delayed	hyt/hyt
	Auditory Orientation	12–25	Delayed	hyt/hyt
	Eye Opening	12–18	Delayed	hyt/hyt
	Ear Raising	14–24	Delayed	hyt/hyt
	Home Orientation	4–22	Delayed	FHT; FHyp
	Grip Strength		Decreased	FPCB, hyt/hyt
MOTOR	Open Field Movements	5–7	Increased	FHyp
	Hyperactivity	14,21,40, 120	Present	FDIOX; FPCB; EC; FHT;NHT; hyt/hyt
	Hypoactivity	14,21,40,120	Present	FPCB; hyt/hyt; FHT
	Walking Pattern Analysis	120	Abnormal	FHT
SENSORIMOTOR INTEG	Swimming	6–23	Accelerated	FPCB; NHyp
	Swim Escape	45–49	Abnormal	hyt/hyt
ADAPTIVE BEHAVIOR	SIMPLE LEARNING			
	Water T-Maze or T-Maze	46–57	Abnormal	FPCB; NHT
	Operant Conditioning	41–72	Abnormal	
	COMPLEX LEARNING			
	Spatial/Water Maze (Morris)	90–107	Abnormal	hyt/hyt
	Passive/Active Avoidance	70–114	Abnormal	FDIOX; FPCB; EC; NHT
	Symmetrical, Spatial, Residential Mazes	35–180	Abnormal	FHT; NHT; NHyp
	Observational	50–60	Abnormal	FHT; NHT
OTHER BEHAVIORS	Audiogenic Seizures/Spinning		Present	FDIOX; FPCB; EC; hyt/hyt
	Hearing Abnormalities		Present	hyt/hyt, NHT

Abbreviations: EC, Endemic cretinism with fetal hypothyroidism produced by iodine deficiency; FDIOX, fetal dioxin exposure; FHT, fetal hypothyroidism; FHyp, fetal hyperthyroidism; FPCB, fetal PCB exposure; hyt/hyt, hyt/hyt hypothyroid mouse; NHT, neonatal hypothyroidism; NHyp, neonatal hyperthyroidism. The data for hypothyroidism cited in this table are from the following references: Eayrs and Levine, 1963; Essman et al., 1968; Davenport et al., 1976a,b; Schalock et al., 1979; Johanson, 1980; Johanson et al., 1980; Narayanan et al., 1982; Strupp and Levitsky, 1983; Overstreet et al., 1984; Comer and Norton, 1985; Adams et al., 1989; Anthony, 1990; Anthony et al., 1993. The following references were cited for hyperthyroidism: Davenport et al., 1975; Stone and Greenough, 1975; Sjoden and Soderberg, 1976. The following references were cited for PCBs and Dioxins: Reviewed in Tilson et al., 1979; Pantaleoni et al., 1988; Van den Berg et al., 1988; Holene et al., 1995.

level have local consequences on neurons, as well as cause disruption of connectivity between neurons in local circuits and between regions of the brain. The basal forebrain, olfactory lobes, hippocampus, and projections to the frontal lobes from associative corticies appear to be particularly affected by changes in thyroid hormone homeostasis (Marinesco, 1924; Rosman, 1975; Rami et al., 1986a,b; Lauder, 1989; Oh et al., 1991).

Neuron differentiation and development of motor function. The age of onset of rodent thyroid function and the time of onset of hyt/hyt hypothyroidism correlate temporally with cerebral neurogenesis of layer V sensorimotor cortex pyramidal neurons and initial pyramidal neuron differentiation (Wise and Jones, 1983). The layer V pyramidal neurons of the sensorimotor cortex give rise to the corticospinal tracts. Corticospinal axonal growth from layer V pyramidal neurons occurs in two phases: rapid, early, long distance elongation and a later, shorter, directed invasion of dorsal horn regions of specific levels of the spinal cord (Jones, 1981; Schreyer and Jones, 1982). The time of final penetration correlates with the onset of forelimb placing, and subsequently with hindlimb placing and the normal forelimb grasping response (Adams et al., 1989). This, too, may be altered in the hyt/hyt mouse which may explain the reflexive motor delays. In humans with SCH, altered, persistent, or asymmetric grasp reflexes reflect altered motor function.

In order to better study the layer V pyramidal neurons, adult and day of birth animals were studied by retrograde and anterograde tract tracing (Figure 4). An estimated 3920 retrogradely labeled corticospinal tract neurons were observed bilaterally in adult +/+ mice following Fluorogold injection of the spinal cord, providing optimal coordinates for anterograde injection and tract tracing.[3] We have now started to define the corticospinal tract development in wild type mice as a prelude to detailed anatomic studies of corticospinal tract development in the hyt/hyt and hyt/+ mice during gestation and in the postnatal period. The events of tract growth and penetration to successively descending levels may be slowed and altered in the hyt/hyt mouse.

The growth of the corticospinal tract into the caudal spinal cord can be determined by retrograde Fast Blue (FB) tracing (Figure 4). When 2% FB was injected into the lumbar enlargement (L4-5) of the spinal cord at PND 0, labeled neurons were found in a number of brainstem nuclei whose descending spinal pathways grow into spinal cord much earlier than the corticospinal tract (Figure 4I,K). The labeling of these brainstem nuclei indicates that the injection and transportation of FB were appropriate. Cortical neurons were not labeled (Figure 4M), suggesting that corticospinal axons have not grown into the lumbosacral cord at PND 0. When injections were made at PND 21, FB labeled cells were found in the layer V of the hindlimb region of the sensorimotor cortex (Figure 4N), indicating that corticospinal axons projected to the lumbosacral cord at or before the age of PND 21.

Alterations in proteins influence neural development. One of the mechanisms by which thyroid hormone influences fetal neurogenesis, migration, process growth, synaptogenesis, and cell death is by altered expression of proteins that regulate these events [e.g. calmodulin, synapsin I, myelin basic protein, microtubule associated proteins (MAPs)] and trophic factors, i.e. neural growth factor and epidermal growth factor (Farsetti et al., 1991; Munoz et al., 1991; Bernal et al., 1992;

3. Holets, V. and Stein, S. (1994). Unpublished observation, University of Miami, FL.

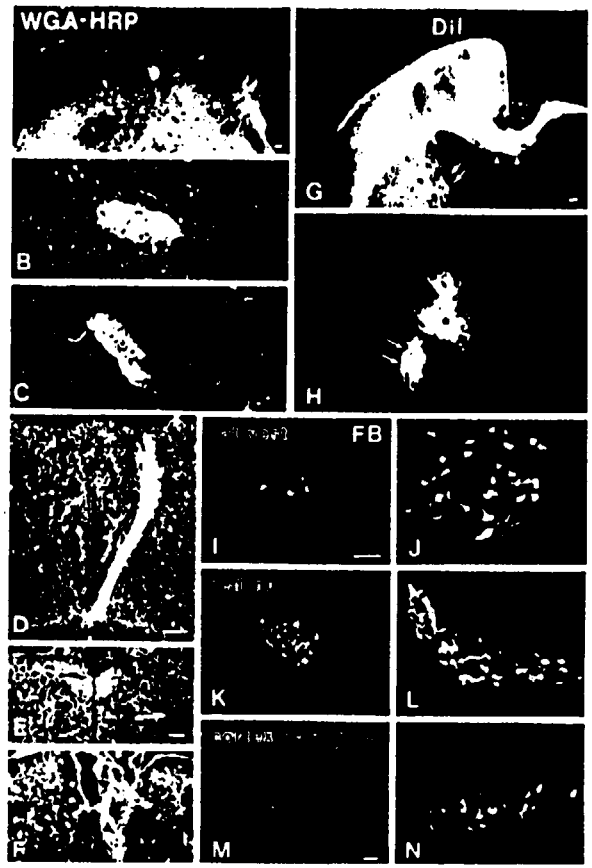

FIGURE 4. Anterograde and retrograde tracing of the corticospinal tracts in day of birth (PND 0) mice. Photomicrographic results of the three tracing methods described. (1) Anterograde tracing with WGA-HRP (A-F): A shows the injection site (i.e., the left sensorimotor cortex) of a PND 0 BALB/cBY mouse. The condensed corticospinal tract (CST) can be followed in the left internal capsule (B), cerebral peduncle (C), and to the pyramidal decussation (D). After the decussation, the CST axons descend dorsally on the right side at the cord-medullary junction (E). A few leading axons were found as far caudally as the cervical enlargement (arrowhead, F). (2) Anterograde diffusion of DiI (G-H): G shows the DiI injection site (asterisks) in the left cerebral cortex of a PND 0 BALB/cBY mouse. Notice the heavily labeled corticostriatal projections to the caudate and putamen (arrows) and corticocortical commissural projections to the cortex on the right (arrowheads). A condensed CST in the internal capsule is clearly seen (H, arrows) with its collateral projections to the adjacent thalamic nuclei (*). The remaining course of DiI labeled CST is similar to that labeled with WGA-HRP. (3) Retrograde tracing with Fast Blue (I-N) in PND 0 (left column) and PND 21(right column) of BALB/cBY mice was compared. In the PND 0 mouse, labeled cells were found in the brainstem nuclei, such as lateral vestibular nuclei (I) and red nucleus (K) whose axons project to the spinal cord earlier than CST, indicating that the injection and retrograde labeling were successful. The lack of labeling in the hindlimb region of the sensorimotor cortex at PND 0 (M) suggests that the CST has not grown into the lumbosacral cord at birth. The FB retrograde labeling of layer V pyramidal cells in the hindlimb region of the cortex was easily found at PND 21 (N), along with the labeling in other brainstem nuclei seen in PND 21 (J, L). All bars are equal to 100 μm. The bar in A can be used for B, C, that in E for F, in G for H, in I for J-L and N.

Giordano et al., 1992; Porterfield and Stein, 1994; reviewed in Biesiada et al., 1996). The hyt/hyt mouse has been used to evaluate the regulation of process growth by a variety of different molecular studies. Thyroid hormone had specific effects on selective genes at differing times in the life cycle. In particular, altered levels in the mRNAs of tubulin isoforms in late gestation were seen when hyt/hyt brains were compared to euthyroid, hyt/+ littermates (Stein, 1994; Biesiada et al., 1996). These alterations may contribute to the resultant abnormalities in corticospinal growth and observed abnormalities in motor behavior.

The hyt/hyt TSH receptor gene mutation. Molecular data pointed to a mutation in the coding region of the TSHr gene as the cause of the TSH hyporesponsiveness. However, Southern blot hybridization of genomic deoxyribonucleic acid (DNA) from hyt/hyt, hyt/+, and +/+ mice using a TSHr probe indicated no gross insertion, deletion, or duplication of the hyt/hyt TSHr gene (Stein, 1994). Sequence analysis of the entire coding region and adjacent noncoding regions (2510 bases in total) of the TSHr revealed only a single C to T transition at nucleotide number 1666 in the hyt/hyt TSHr. This CCG to CTG transition changes a proline to a leucine at amino acid 556 in transmembrane domain IV. This proline is highly conserved in all other glycoprotein hormone receptors and in at least 82% of other G protein-coupled receptors. The presence of both C and T at position 1666 in the hyt/+ euthyroid mice affirms the observed autosomal recessive inheritance of the hyt gene and the classification of the hyt/+ mice as heterozygotes (Adams et al., 1989; Stein et al., 1989). Therefore, one normal allele may be sufficient for normal TSHr function.

Binding and localization of the hyt/hyt TSHr. The mutation in the hyt/hyt mouse TSHr was the first demonstration of a naturally occurring mutation in the TSHr with functional consequences. The Pro to Leu replacement in the hyt/hyt TSHr may abolish ligand binding by a structural effect on the binding regions of the TSHr (the extracellular domain) or by interference with translation, trafficking, or appropriate insertion of the receptor on the cell membrane (Stein, 1994). These observations suggest that the hyt/hyt mutation does not interfere with the transcription of TSHr mRNA, or of transport, processing, targeting, and plasma membrane insertion of the TSH receptor. However, the final verdict on this will come from fluorescent antibody cell sorter analysis with mutant and wild type TSH receptors, which may detect a subtle trafficking defect. With regard to the latter, our studies indicate that the extracellular domain, including its most terminal extent, achieves an extracellular position with respect to the plasma membrane. These observations suggest that the obliteration of TSH binding to the hyt/hyt TSHr is related to a change in tertiary structure of TSHr brought on by the conversion of a conserved and functionally important proline to leucine. This also suggests that transmembrane domain IV is critical for normal TSH binding to the TSHr. The abolition of binding prevents the normal transduction of a TSHr-cAMP response, leading to TSH hyporesponsiveness and a significant reduction in thyroid hormone synthesis by the hyt/hyt thyroid gland. This relationship is depicted in Figure 5.

Given the applicability of the hyt/hyt mouse to human SCH, and the motor and cognitive signs common to both, the hyt/hyt animal is a useful model for delineating thyroid hormone mediated neurobiology throughout the life cycle. This model can be applied to other situations where thyroid hormone disruption is a postulated mechanism of neurotoxicity (see below).

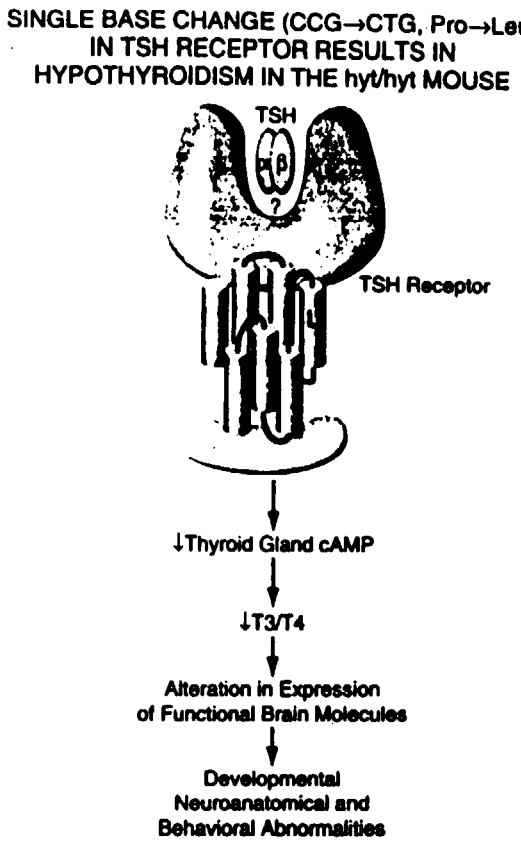

FIGURE 5. Model of sporadic congenital hypothyroidism, the hyt/hyt mouse. The hyt/hyt mouse has an autosomal recessive, fetal onset, severe hypothyroidism that persists throughout life. The hypothyroidism is due to hyporesponsiveness of the thyroid gland to TSH. This is attributable to a point mutation of C to T in the nucleotide at position 1666, resulting in the replacement of a Proline to Leucine at amino acid 556 in transmembrane domain IV of the G protein-linked TSH receptor. This mutation leads to a reduction in all cAMP regulated events, including thyroid hormone synthesis. The diminution of T_3 and T_4 in the serum and organs, including the brain, also leads to alterations in the timing and level of expression of critical brain molecules, i.e. selected tubulin isoforms, MAPs, and myelin basic protein, as well as changes in important neuronal cytoskeletal events, i.e. microtubule assembly and axonal transport. In the hyt/hyt mouse, fetal hypothyroidism leads to reductions in Mβ5, Mβ2, and Mα1 mRNAs and proteins and tubulin isoforms which comprise the microtubules. These molecules are localized to layer V pyramidal neurons in the sensorimotor cortex, a site of differentiating neurons, as are thyroid hormone receptors. These molecular abnormalities in specific cells at specific times in development may contribute to the observed neuroanatomic abnormalities, i.e. altered neuronal process growth and maintenance, synaptogenesis, and myelination in hypothyroid brain. Abnormal neuroanatomic development in selected brain regions may underlay the abnormalities in reflexive, locomotor, and adaptive behavior in the hyt/hyt mouse and other hypothyroid animals (reviewed in detail in Biesiada et al., 1996). (With permission from Stein et al., 1991d; Biesiada et al., 1996.)

Effects of PCB and Dioxin Exposure on Animal Behavior

Behavioral studies of rodents exposed *in utero* to PCBs and dioxins are presented, in part, in Table 4. In PCB exposed rodents, grip strength was reduced (Sauer et al., 1994), reflecting impaired corticospinal tract functioning, but cliff avoidance and negative geotaxis were not delayed (Overmann et al., 1987). Hypoactivity and hyperactivity were observed (Tilson et al., 1979; Lilienthal et al., 1990; Holene et al., 1995), as well as altered swimming behavior and complex passive/active avoidance abnormalities (Pantaleoni et al., 1988). Taken together, fetal PCB and dioxin exposure may alter certain reflexive, locomotor, and adaptive behaviors that depend on normal motor development. Similarly, the spinning syndromes observed (Chou et al., 1979) may be caused by anterior horn cell abnormalities. Additional analysis of these studies as well as future behavioral studies will be useful for determining if the behavioral effects of endocrine disrupters are thyroid specific.

Human and rodent behavioral and anatomic studies have shown that the prefrontal cortex is a common target for thyroid hormone and endocrine disruptors. Dysfunction of the prefrontal cortex may be manifested by attentional disorders, apathy or depressed mood, manic behavior, expressive speech difficulties, alterations in social behavior, disorders of planning (including motor plans), disorders of integrating sensory and other stimuli, and abnormalities in sequence testing, card sorting, and verbal fluency (Strub and Black, 1988). The cholinergic basal forebrain system, a pathway within the prefrontal cortex, has been shown to be dependent on homeostatic levels of thyroid hormone during the late gestational and early postnatal period in rats (Oh et al., 1991). This study demonstrated that hypothyroidism resulted in a reduction in process growth with a delay in neurotransmitter expression that was not reversible.

The only animal models showing thyroid hormone specific (prefrontal cortex) alterations in behavior when exposed to PCBs and dioxins are monkeys. In 4–6 year-old rhesus monkeys prenatally exposed to low levels of Aroclors (Aroclor 1016 and 1248), a significant decrement occurred in discrimination-reversal learning and delayed spatial alternation, which when combined suggest a lesion of the orbital prefrontal cortex that may involve dopaminergic projection pathways (reviewed in Schantz et al., 1991). Although Aroclor may alter maternal and fetal thyroid hormone levels, this was not studied in these monkeys. Hyperactivity has also been shown in rhesus monkeys (Bowman et al., 1981). In addition, social behavior, which is a frontal lobe function, was altered in rhesus monkeys born to mothers with chronic intake of dioxins. The progeny of exposed monkeys demonstrated alterations in peer group related social behavior (Schantz et al., 1992), which might be classified as prefrontal or frontal in origin. The selective nature of these frontal lobe abnormalities and the observation of key frontal lobe and dopaminergic pathway targets for thyroid hormone suggest that certain PCB effects may have a relationship to thyroid hormone.

Regulation of Brain Mitochondrial Genes in Rodent Models: ATPase 6

The adult brain, as compared to the liver and neonatal brain, has been thought to be relatively insensitive to thyroid hormone. However, prominent behavioral and anatomic effects of thyroid hormone in the juvenile and adult brain have been observed (Iniquez et al., 1993). Because of the potential dichotomy of effects of thyroid hormone in brain and liver, we have studied the effects of different thyroid states on gene expression in rodent liver and brain. The major purpose of the study was to establish that the transition from the hypothyroid to euthyroid to hyperthyroid states in the adult rodent has significant effects on the brain and liver at the level of gene expression.

Adult Sprague Dawley rats were made hyperthyroid by intraperitoneal injection of T_4 and were used with euthyroid age-matched females. Total RNA was isolated from hyperthyroid rat liver and used for construction of a complementary deoxyribonucleic acid (cDNA) library. This library was screened with sequential colony hybridization to identify sequences that might be regulated by changes in thyroid hormone level. Single stranded mixed cDNA probes from euthyroid and hyperthyroid rat liver and brain were used to screen the library, colonies being identified by grid coordinate (Figure 6). In comparing specific mRNAs in the euthyroid to hyperthyroid liver and brain, the majority of colonies were not significantly changed in abundance. Eight percent of the liver colonies did show differences, with increases in 9A6, 1F5, and 7F5, and decreases in 5C5. The number of colonies that showed a greater than ten-fold change was less than 1% of the total colonies. In the brain, 2.5% of mRNAs common also to liver demonstrated changes in abundance. This would predict 12–70 brain sequences common to liver that are regulated by thyroid hormone in the adult brain. Of these regulated mRNAs, one (9A6) was chosen for study across tissues at a variety of different thyroid hormone levels.

To do so, 9A6 was evaluated in euthyroid and hyperthyroid rat and mouse brain and liver on northern gel (Figure 7B) and slot blot hybridization (Figure 7C). 9A6 mRNA is present at higher abundance in rat and mouse cerebral cortex compared to thalamus, cerebellum, and brainstem (Figure 7A). Unlike the euthyroid to hyperthyroid transition in rat brain, the 9A6 mRNA was depressed 3–5-fold in abundance in the hyperthyroid mouse brain compared to the euthyroid rat brain (Figure 7B). No change for 9A6 mRNA was observed in the mouse liver.

This was followed by analysis of 9A6 mRNA abundance in day of birth hyt/hyt versus hyt/+ mice. To assess the effects of hypothyroidism, hyt/hyt and hyt/+ adult and neonatal mice were used with BALB/cBy progenitor strain controls. 9A6 mRNA was present in day of birth cerebral cortex and total brain of hyt/hyt and hyt/+ mice. This reflects late gestation expression of this mRNA in the mouse fetus. This mRNA was also expressed in similar abundance at five and seven days after birth. No change in abundance was noted in the 9A6 mRNA between the day of birth hyt/hyt and hyt/+ littermates, suggesting that this mRNA is not regulated in the transition from the hypothyroid to euthyroid state in late gestation fetal cerebral cortex and total brain.

Sequence analysis of the 9A6 cDNA was performed to try to identify this mRNA. The sequence is shown in Figure 8. Based on this analysis, the cDNA was homologous to the mitochondrial genome product ATPase 6. The ATPase 6 gene is one of 13 genes on the mitochondrial genome and encodes for one of three critical subunits in the Fo portion of ATP synthase. ATP synthase is composed of two parts, the ATP catalytic portion (F1) and the membrane H+ pore (Fo). The five F1 subunits are encoded by nuclear genes as is one of the three Fo subunits. The dual localization of the genes for these subunits allows for complex regulation and sites for disruption by changes in thyroid hormone level (Izquierdo et al., 1995). Alteration in 9A6 mRNA or ATPase 6 by thyroid hormone may have significant effects on energy metabolism.

Thus, 9A6 represents a gene product that is regulated by thyroid hormone across species, tissues, and ages. Physiological hypo- and hyperthyroidism have distinct effects on a subset of the total genes in adult rodent liver and brain. Gene expression was not turned off completely by hyper- or

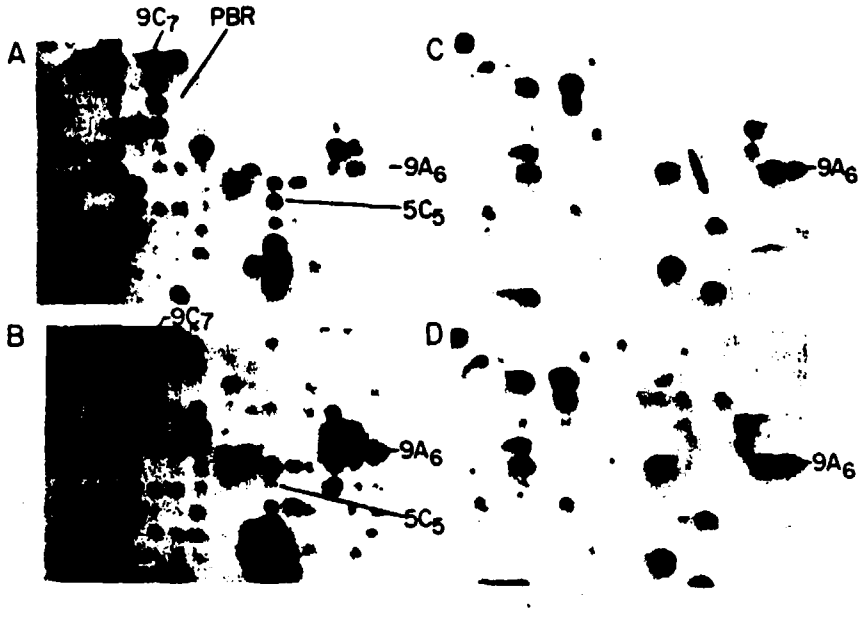

FIGURE 6. Sequential colony hybridization of liver cDNA library with mixed cDNA probes. Equal amounts and time of exposure were used for each cDNA probe on the same 541 filter sheet to which the colonies were affixed. A portion of the total sheet for each probe is shown, with important cDNAs to specific thyroid hormone regulated mRNAs noted. A-mixed euthyroid (ET) liver cDNA probe; B-mixed hyperthyroid (HT) liver cDNA probe; C-mixed euthyroid (ET) brain cDNA probe; D-mixed hyperthyroid (HT) cDNA probe. Equal amounts of rat liver and brain euthyroid and hyperthyroid mixed cDNA probes, 10^7 cpm/mL, made by reverse transcription of total RNA were sequentially added to the 541 papers and hybridized for 65 hours at 41°C and washed. Autoradiography was for 18 hours for each sheet. Probes were washed off the filters with a single sodium hydroxide wash and then reautoradiographed after the wash for 72 hours to look for complete probe removal before addition of different cDNA probes. The same 541 sheet was then used for the next colony hybridization with the euthyroid or hyperthyroid cDNA probe. Visually, 9A6 was increased and 5C5 was decreased. Autoradiographs representing the different probes were analyzed by visual analysis and by quantitative analysis. For the quantitative analysis, the autoradiographs were digitized on an Optronic P1700 rotating drum scanner, and then the autoradiographic image was processed and smoothed with an image processing program[4] on a Vax 11/780. The background was located and fit to a smooth polynomial function and the background was subtracted from each colony density. The density of each colony was then integrated and plotted numerically with a Calcomp plotter. This information was used to determine the percentage of mRNAs common to liver and brain that might be altered by hyperthyroidism, to identify cDNAs homologous to specific mRNAs that might be altered by thyroid hormone, and to identify cDNAs homologous to specific mRNAs that are unique for liver or common to liver and brain.

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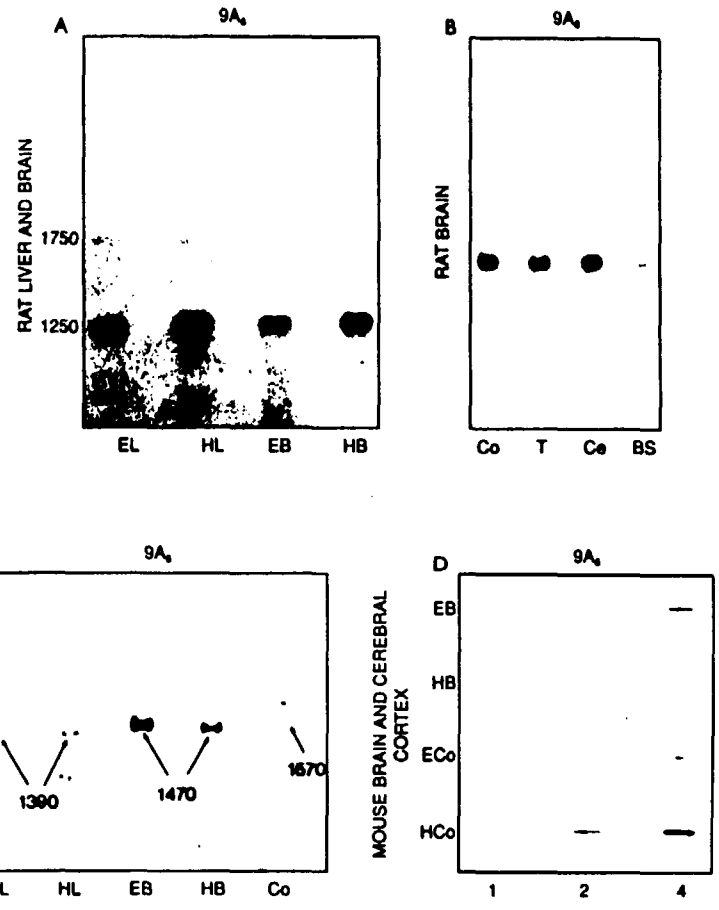

FIGURE 7. Northern gel and slot blot hybridizations of 9A6 cDNA to euthyroid (E) and hyperthyroid (H) adult rat and mouse liver (L) and mouse total brain (B) and individual brain regions [Co (Cortex); T (Thalamus); Ce (Cerebellum); Bs (Brainstem)] (Panels A,B,C). *Slot Blot Hybridization* of 9A6 cDNA to 1,2, and 4 μg of total RNA from mouse euthyroid (E) and hyperthyroid (H) total brain (B) and cerebral cortex (CC) (Panel D). Putative sizes of mRNAs are shown. Actin was used as a negative control (not shown). Panel A: 9A6 mRNA is increased in transition from the euthyroid to hyperthyroid state in the rat liver and brain. Panel B: 9A6 mRNA is present at higher abundance in rat cerebral cortex, cerebellum, and thallamus compared to brainstem. Panel C: Unlike the euthyroid to hyperthyroid transition in rat brain, the 9A6 mRNA was depressed 3-5-fold in abundance in the hyperthyroid mouse brain compared to the euthyroid mouse brain. No change was observed in the mouse liver. The numbers on this panel represent calculated molecular weight. Panel D: In the mouse cerebral cortex and total brain, this is a high abundance to high middle abundance mRNA. 9A6 demonstrates about a five-fold lower mRNA abundance in hyperthyroid total brain versus euthyroid total brain. However, in the euthyroid cerebral cortex versus hyperthyroid cerebral cortex, an increase of five-fold in mRNA abundance is seen. The numbers on this panel represent the number of micrograms of total RNA that were hybridized to the 9A6 cDNA.

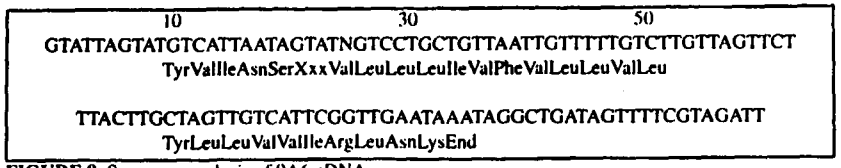

FIGURE 8. Sequence analysis of 9A6 cDNA.

hypothyroidism; rather, we have observed perturbations in the basal level of expressed 9A6 mRNA. 9A6 is one example of thyroid hormone regulation of a selective and unique set of genes that code for functionally important proteins at different times and in different tissues.

THYROID HORMONE, ENERGY PRODUCTION, AND MITOCHONDRIAL GENES

The linkage between thyroid hormone, cellular energy metabolism, thermogenesis, and the mitochondria has been evaluated by numerous studies (Sterling, 1986; Hood et al., 1992; Luciakova and Nelson, 1992). Both thyroid hormone and PCBs may affect oxidative phosphorylation. PCBs have been postulated to uncouple oxidative phosphorylation (Lans et al., 1990; Narasimhan et al., 1991). Mitochondria contain their own DNA and are capable of synthesizing their own proteins separately from the nuclear transcriptional and translational machinery. The thirteen proteins are all enzymes or comprise the translational machinery involved in the respiratory chain complex, the common pathway from which glucose metabolism and fatty acid oxidation generate ATP. Hence, a disruption in this system may have significant effects on the generation and storage of energy. The mitochondrial matrix is surrounded by a relatively impermeable membrane, thus fatty acid substrates must be actively transported across it via carnitine palmitoyltransferase. Once these substrates have entered the matrix, they are further metabolized: pyruvate is transformed to acetyl Co-A by the action of the pyruvate dehydrogenase complex, and fatty acids are transformed to the same via beta oxidation. Depending on the size of the fatty acid, any of three dehydrogenases may be involved in beta oxidation: Short chain acyl-Co-A dehydrogenase, medium chain acyl-Co-A dehydrogenase, or long chain acyl-Co-A dehydrogenase. The resultant acetyl Co-A is oxidized through several steps in the Krebs Cycle to reducing equivalents which flow through the five complexes of the respiratory chain in a series of oxidation/reduction reactions which ultimately lead to the generation of ATP and water.

The expression of many of these metabolic enzymes is regulated by thyroid hormone. Thyroid hormone has been shown to regulate the production of Cox I, Cox III, cytochrome c1, cytochrome c oxidase, and b-F1-ATPase mRNAs in the respiratory chain (Hood et al., 1992; Luciakova and Nelson, 1992; Izquierdo et al., 1995; Stevens et al., 1995; Klingenspor et al., 1996). Levels of three mitochondrial cDNAs in brain (12S and 16S rRNAs and cytochrome c oxidase subunit III) were reduced by hypothyroidism and restored by administration of T_3. Likewise, in the brain, gene products derived from nuclear transcription and then transported for contribution to the mitochondrial protein complexes (Cox IV, Cox VIc) were also regulated by thyroid hormone (Vega-Nunez et al., 1995). In addition, upstream metabolic enzyme machinery was regulated in part by thyroid hormone, i.e. carnitine palmitoyltransferase-1, adenine nucleotide translocase, and glycerol 3-phosphate dehydrogenase which provide substrates to the mitochondria for oxidative phosphorylation (Mynatt et al., 1994; Dummler et al., 1996).

These observations indicate that thyroid hormone influences gene expression at the level of the mitochondrial and nuclear genomes, suggesting that some extranuclear effects of thyroid hormone may be transcriptional in nature. Some effects of thyroid hormone on the mitochondria, particularly in the brain, may be on genes that control products critical for the production of ATP, such as Cox I and III and ATPase 6. An important hypothesized mechanism of PCB and dioxin toxicity is the alteration of the levels of brain thyroid hormone, leading to changes in critical mRNAs and their protein products, including mitochondrial cytochrome oxidase and ATPase 6. Additional studies will be useful in helping to define the differential regulation of ATPase 6 across tissues, species, and in different thyroid states. The mechanisms by which thyroid hormone regulates individual or multiple mitochondrial genes in a coordinate fashion and any possible interference by endocrine disruptors may then also be defined.

The Clinical Significance of Alterations in Mitochondrial Genes

Patients with a number of inherited disorders of the mitochondria caused by mutations in specific mitochondrial genes or genes involved in the breakdown of sugars, proteins, and fatty acids may be more susceptible to endocrine-disrupting agents and alterations in thyroid hormone. These include Leigh Syndrome, MELAS (myopathy, encephalopathy, lactic acidosis and stroke-like episodes), MERRF (myoclonus epilepsy with ragged red fibers), striatal necrosis, Alper Syndrome, and inclusion body myopathy. Leigh syndrome [subacute necrotizing encephalomyopathy (SNE)] is a neurodegenerative disorder, generally presenting in infancy with lactic acidosis, progressive developmental delay, ataxia, seizures, and brainstem dysfunction, including respiratory abnormalities, hearing loss, nystagmus, and ophthalmoplegia. Four mitochondrial point mutations have been described in this disease. Three have occurred in the gene encoding for ATPase subunit 6 (Shoffner et al., 1992; Santorelli et al., 1993) and one in cytochrome oxidase III (complex IV) and in complex I. These same mutations have also been identified in other mitochondrial disorders including NARP (neurogenic muscle weakness, ataxia, and retinitis pigmentosa), a milder form of SNE associated with a lower percent of mutant mitochondria (Holt et al., 1990). In addition to mutations in the mitochondrial genome, this disorder is also associated with mutations in nuclear genes which are targeted for mitochondrial delivery, or with abnormalities in upstream metabolic steps (abnormalities of the pyruvate dehydrogenase complex, reviewed in DiMauro and DeVivo, 1996). Familial bilateral striatal necrosis also has been associated with a mutation in the gene for ATPase 6 at nucleotide 9176 (Thyagarajan et al., 1995).

In addition to regulation of these mitochondrial genes, thyroid hormone can also cause proliferation of mitochondria. The ragged red fibers seen in many of these mitochondrial disorders represent proliferation of mitochondria (DiMauro et al., 1990). Thyroid hormone abnormalities may contribute to the pathology seen in the mitochondrial disorders. Similar brain regions and neural tissues are affected in thyroid disorders and mitochondrial disorders, i.e. the motor system, brainstem, basal ganglia, muscle, and peripheral nerve.

In many of the mitochondrial disorders, the affected individuals appear to be neurologically intact until a metabolic stressor occurs, such as an acute infection. The individuals most clearly at risk are those with the partial forms of these disorders, who may be functioning well until increased demands are placed on the metabolic system. In these marginally compensated

individuals, exposure to any substance which would downregulate the remaining wild-type mitochondrial genes could lead to an inability to maintain normal brain and muscle energy production. This could potentially occur with PCB or dioxin exposure *in utero* or throughout the life cycle.

RESEARCH GAPS AND FUTURE DIRECTIONS

Although there appears to be a strong suggestion that PCBs and dioxins interfere with thyroid hormone secretion and metabolism, there are issues related to current published research which prevent a clear assessment of human risk. The animal studies were performed in a variety of different species with a variety of different PCB compounds. Since different species depend on slightly different methods of storing, metabolizing, and using thyroid hormone, and since different compounds may act as agonists, partial agonists, or antagonists, it is difficult to assess how the studies may apply to humans. Furthermore, it is not yet known if there is a certain threshold level of thyroid hormone below which there is irreversible damage to the fetal brain or if the effect is more of a continuum. To date, studies comparing biochemical findings to histological changes in the brains of the exposed animals have not been performed. If PCBs do lower T_4 concentrations in the brain during the crucial period of pyramidal tract development or neuronal differentiation, it would be important to perform these experiments to assess whether this decrease has neuroanatomic effects even with low or moderate PCB doses.

Since multiple contaminants, known and unknown, may coexist in the same environment, it is difficult to tease out effects attributable solely to PCB or dioxin exposure. In addition, these compounds have several other proposed modes of action, other than those of thyroid hormone disruption. They have been hypothesized to exhibit neurotoxicity through interaction with the arylhydrocarbon receptor and/or through diminution of dopamine synthesis.

Understanding of the potential linkage between thyroid hormone and endocrine disruptors and the development of the nervous system is evolving. As focused on in this review, neurologic, behavioral, and neuropsychologic abnormalities in humans and rodents reflect selective effects of thyroid hormone on the development and maintenance of many sites within the nervous system, particularly the motor system and the cerebral cortex. Neuronal process growth and connectivity and their molecular substrates form one basis for the neuroanatomic and clinical disorders of thyroid hormone. These thyroid mediated effects should provide a template for future studies to assess the putative effects of endocrine disruptors and to substantiate their role in disrupting thyroid hormone regulated events.

Certain clinical abnormalities, such as diminution in head circumference and neonatal motor delay, are seen with both thyroid hormone deficiency and with PCB exposure. However, the neurological measurement tools are not consistent across studies to allow firm conclusions or assessment of specific risk to be drawn about these compounds. Additional human studies are needed using standardized, broad-based test instruments and neurologic examination in

combination with simultaneous thyroid hormone measurements in regions of both low and high exposure. The populations at risk, including those with thyroid hormone disorders *in utero* and metabolic disorders, particularly involving the mitochondria, deserve particular attention in future studies of endocrine disruptor and thyroid hormone pathophysiology.

Although neurologic and neuropsychologic studies of adults exposed to PCBs and dioxins are limited, thyroid hormone affects the brain throughout the life cycle. Therefore, a potential link between these endocrine disruptors and thyroid hormone must be based on additional evaluations in children and adults.

It is clear that additional work in animal models is also required to elucidate common mechanisms by which thyroid hormone and PCBs or dioxins influence molecular and anatomic brain development and disrupt mitochondrial function. Neurochemical abnormalities in certain brain regions have been seen in Aroclor exposed fetal rats with low brain thyroid hormone levels (Morse et al., 1996a,b). To take this further, the hyt/hyt mouse and other animal models of thyroid hormone disease will be useful for evaluating the effects of putative endocrine disruptors and their relationship to thyroid hormone through comparison of behavioral, neuroanatomic, biochemical, and molecular differences in endocrine disruptor exposed and unexposed hypothyroid mice and normal mice. Additional clinical and basic research is required to evaluate potential promising common mechanisms for the actions of thyroid hormone and endocrine disruptors in the brain. This will require joint, coordinated multidisciplinary and multinational efforts by basic and clinical scientists, physicians, governments, and industry.

ACKNOWLEDGMENTS

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EFFECTS OF PESTICIDES AND TOXIC SUBSTANCES ON BEHAVIORAL AND MORPHOLOGICAL REPRODUCTIVE DEVELOPMENT: ENDOCRINE VERSUS NONENDOCRINE MECHANISMS

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Exposure to toxic substances or pesticides during critical perinatal developmental periods can alter reproductive and central nervous system (CNS) function in a manner that does not compromise the growth and viability of the fetus but causes functional alterations that become apparent later in life. While some "CNS/behavioral teratogens" are mutagenic or alter cell division, other chemicals produce alterations of CNS development via endocrine-mediated mechanisms. The following discussion will focus on studies conducted primarily in our laboratory that describe how pesticides and toxic substances alter development of the reproductive and central nervous systems as a consequence of organizational or activational exposures. Abnormal behavior and morphology can result from exposure to endocrine-disrupting toxicants by altering organization of the CNS during critical stages of life or activation of behavior after puberty. Some of the toxicants that alter rodent sexual differentiation include xenoestrogens, antiandrogenic pesticides, and dioxin-like toxic substances. Chemicals that alter sex-linked nonreproductive and reproductive CNS development via nonhormonal mechanisms are also discussed in order to demonstrate that multiple mechanisms of action are involved in the development of

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2. Abbreviations: B, busulfan; CNS, central nervous system; DES, diethylstilbestrol; DHT, dihydrotestosterone; E_2, estradiol; EDC, endocrine-disrupting chemical; EDS, Ethane-1,2-dimethanesulfonate; ER, estrogen receptor; ESC, ejaculated sperm count; GD, gestational day; INAH, interstitial nuclei of the anterior hypothalamus; LE, Long Evans; PCB, polychlorinated biphenyl; PD, post-dosing; PND, postnatal day; PO-AHA, preoptic-anterior hypothalamic area; SDN-POA, sexually dimorphic nucleus in the preoptic area; T, testosterone; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; TP, testosterone propionate.

3. Key words: behavior, CNS, endocrine-disrupting chemicals, hypothalamus, reproductive development, sex dimorphism.

4. Disclaimer: The research described in this article has been reviewed by the National Health and Environmental Effects Research Laboratory, U.S. Environmental Protection Agency, and approved for publication. Approval does not signify that the contents necessarily reflect the views and policies of the Agency nor does mention of trade names or commercial products constitute endorsement or recommendation for use.

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